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NATURAL KILLER LYMPHOCYTE MEMBRANE ANTIGENS INVOLVED IN ALLORECOGNITION

Kevin Elisabeth Bishoff

Yale University

1994

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Natural Killer Lymphocyte Membrane Antigens Involved in Allorecognition

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by
Kevin Elisabeth Bishoff
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NATURAL KILLER LYMPHOCYTE MEMBRANE ANTIGENS INVOLVED IN ALLORECOGNITION TARGETING. Kevin E. Bishoff, Jeffrey R. Bender. Section of Cardiovascular Medicine, Department of Internal Medicine, and the Molecular Cardiobiology Program, Boyer Center for Molecular Medicine, Yale University, School of Medicine, New Haven, CT.

It has been recently established that natural killer (NK) lymphocytes have alloantigen recognition capabilities, although the membrane receptor(s) involved is unknown. In the current study, I attempt to characterize such receptors, utilizing an immortal B cell line (RPMI8866) which drives the clonal expansion of antigen-specific NK cells. My hypothesis is that NK cells have a receptor structure that enables them to recognize and specifically lyse these targets.

Murine monoclonal antibodies (mAbs) specific for NK cells were generated and screened by flow cytometry for NK cell specificity and effect on NK cell function. Utilizing RPMI8866-stimulated NK cells as effectors, 2 mAbs (1F7 and 1F8) were defined which partially inhibit lysis (54% and 36% inhibition, respectively, at a 33:1 effector to target ratio) of the stimulating RPMI8866 cell line but not the classical NK-sensitive target K562 at any effector to target ratio.

These results support the hypothesis that NK cells have surface receptors involved in specific target recognition and describe two mAbs which appear to recognize such structures.

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INTRODUCTION

Natural killer (NK) cells are a bone marrow derived subset of lymphocytes found in blood and lymphoid tissues (1). NK cells comprise 1% to 25% of cells in the peripheral blood of humans (2). Because they appear morphologically as large cells with numerous cytoplasmic granules, NK cells are also called large granular lymphocytes (LGLs) in the literature. In a manner analogous to cytotoxic T lymphocytes (CTLs), NK cells kill their targets by granule exocytosis and secretion of a cell toxin. However, unlike CTLs, NK cells do not require prior contact with target antigens to develop cytolytic capacities or cytokine responsiveness (1). The name 'natural killer' refers to this ability to lyse certain cells without prior sensitization or restriction by major histocompatibility complex (MHC) class I or II gene products (2-4). That is, they mediate cytolytic reactions that do not require expression of class I or class II MHC molecules on the target cells (3).

Although there is a subset of T cells which can also lyse their targets in an MHC-unrestricted fashion, NK cells are distinct from this subset in that they lack the surface molecule CD3 and the T cell receptor (TCR) for antigen recognition. Unlike B and T cells, respectively, NK cells do not undergo Ig or TCR rearrangement, which, via the creation of a variety of surface receptors, enables them to specifically recognize antigens. Lymphocytes without sIg or TCR perform "nonspecific" lethal action on various susceptible cell types but, in general, were previously considered unable to recognize antigen(s).

From its discovery in 1974 until very recently, identification of the NK cell based solely on its morphologic characteristics, and on certain of its known functions, some which are shared with other cell types, such as monocytes, macrophages and activated T cells, posed a major limitation to NK cell analysis. Monoclonal antibodies (mAbs) to cell surface molecules can phenotype NK cells, differentiating them from other lymphoid cells with common cytotoxic activity or morphology. Surface antigens expressed on human NK cells include class I, CD2, CD7, CD8, CD11a, b, c/CD18 (LFA-1, Mac-1, p150,95),

CD16 (the NK cell Fc receptor for IgG), CD56, and CD57 (5). Although none of these antigens is unique to NK cells, the combination of CD16 and CD56 on a cell lacking the T cell-associated CD3 molecule, is thought to be definitive of the NK cell.

The clinical relevance of the NK cell is still a matter of debate as its function and purpose in normal immunity is not completely understood. Given the lag period between exposure to foreign materials and the development of specific immunity, NK cells were initially thought to be a part of a primary broader-range defense system that can act almost immediately until specific immunity develops (4). However, from in vitro experiments, it has been inferred that NK cells produce lymphokines including interleukin 2 (IL-2) and interferon alpha, and serve in a regulatory capacity to the adaptive immune system and to hematopoiesis, in addition to confering 'natural' resistance against tumors, microbes, fungal, and parasitic agents (6,7). Moreover, it is not known whether all these functions can be mediated by the same cells or whether distinct functional subsets within the NK cell population have different activities (7). Since NK cells have been shown to secrete a variety of lymphokines, it is also not known which of these functions is mediated by direct cytotoxicity and which is mediated through lymphokine secretion (7).

NK cells are able to kill virally infected cells and malignant transformed cells *in vitro*, however they have not been found in inflammatory infiltrates associated with viral infection or tumor (1). The great potential of clinical benefit from understanding NK cell function stems from the wide range of possible targets of NK cell killing, and perhaps that there exist functions beyond cytolysis, including target cell activation and amplification of immune responses.

Early work with NK cells completed in animals revealed their significance in the immune surveillance against the establishment of primary tumors, as well as in controlling the spread of distant metastases (8). IL-2 in vivo and in vitro enhance NK cell cytolytic activity, enabling them to lyse tumor-cell targets that are resistant to unstimulated NK cells (8). Not only the range of tumor cell

recognition, but also the intensity of killing, was shown to highly increase after culture of NK cells in IL-2 containing media (9). In addition, NK cells can be induced to proliferate by high concentrations of IL-2 (1). The effectiveness of these lymphokine-activated killer (LAK) cells has been evaluated in a series of clinical trials in which patients received recombinant IL-2 alone or in conjunction with LAK cells generated in vitro. The metastatic tumors, most often metastatic renal-cell carcinoma or melanoma, regressed in some of the patients who were treated with LAK cells or IL-2 (8).

A number of studies in animals have identified a potentially important role for NK cells in the immune response to certain viral infections (8). In these experiments, NK cells responded rapidly to a viral challenge and mounted both a proliferative and a cytolytic response 4-6 days before a T cell response could be mobilized. There is a little evidence of such a role in humans. Partial deficiencies of NK cells have been documented in a variety of clinical circumstances, including Chediak-Higashi syndrome, leukocyte-adhesion-molecule (CD11/CD18) deficiency, X-linked lymphoproliferative disorder (X-LPD), and the chronic fatigue syndrome. Unlike patients with X-LPD, in whom the NK cell defect is subsequent to EBV infection and might be induced by the virus, the NK cell-deficient patients had a history of repeated viral infection before EBV (6).

In the few patients who have a selective absolute deficiency of NK cells, there is a prevalence of viral pathology. Biron et al described one 17-year-old woman with a total deficiency of NK cells, but normal T and B cell function, who had life-threatening infections with three common herpesviruses: varicella, cytomegalovirus, and herpes simplex virus (8). Eventually specific T and B cell responses could be mobilized, but only after the one to two week time period required for recognition and amplification of these responses. From these data, it appears that NK cells, together with interferon and other natural resistance mechanisms, represent the first line of defense against infection by certain viruses.

Their most impressive, and histologically supported, clinical function may be in the initiation of graft-versus-host disease (GVHD)

in the recipients of allotransplants. Postulated as central to the phenomenon of allograft rejection are the interactions between lymphocytes and microvascular EC, the initial allogenic barrier with which circulating host lymphocytes contact donor tissue in vascularized allografts (10). NK cells bind avidly to (10), induce the appearance of class II MHC antigens on (11), and promote striking morphologic alterations in microvascular EC (12) *in vitro*. Integral membrane protein lymphocyte function-associated antigen-1 (LFA-1) plays a major role in lymphocyte binding to microvascular EC (10), and consequently, induction of EC surface class II expression (11), which is viewed as a sign and a major stimulus of the rejection process (10). The surface density of LFA-1 on NK cells is approximately five times greater than that on $CD4^+$ cells (13).

In vivo data obtained mainly in the mouse suggests that NK cells are involved in the rejection of bone marrow transplants (14). NK cells are the effectors of the genetically restricted hybrid resistance to parental grafts (12). In humans Lopez et al. found an association between high pretransplantation NK cell activity in the recipient against HSV-1 infected target cells and incidence of GVHD after bone marrow transplantation (6). LGLs have been found in fine-needle aspirates from kidney transplant patients in the beginning and early stages of rejection, where their corresponding peripheral blood levels were much lower (15). However, the expression of CD16 and CD56 on these LGLs was not reported.

NK cells are found inside rat renal allografts during rejection. They appear, with strong cytotoxic activity, and disappear *in situ*, prior to the arrival of cytotoxic T lymphocytes (14). From these data, the authors hypothesized that NK cells may have a role in the maturation of the CTL, possibly acting as primary cells for CTL. Furthermore, the presence of NK cells in the renal allograft was simultaneous with their depletion from the spleen, suggesting that they are spleen-derived and not synthesized *in situ*. However, the surface markers on these cells were not characterized.

Allogeneic lymphocyte cytotoxicity, the rapid destruction of i.v. injected allogeneic lymphocytes upon entering the host's lymphoid tissue, may be another nonadaptive immune response in which NK

cells play a role (16). Fossum and Rolstad reported that pretreatment with anti-NK cell antibodies (anti-asialo GM_1 and MRC OX-8) profoundly reduced the proportion of allogeneic cells phagocytozed by interdigitating cells (IDC). Additionally, the rate of killing of allogeneic lymphocytes differed substantially between cervical and axillary lymph nodes and this difference correlated with the densities of anti-asialo GM_1^+ cells in these nodes. *In vitro* studies with cell suspensions rich in IDC from peripheral lymph, but poor in NK activity, could not demonstrate allogeneic lymphocyte cytotoxicity (16).

There is more than one mechanism by which NK cells recognize and kill their target. First, and most clearly understood, NK cell killing can be directed by opsonization of target cells with antibody. These IgG molecules are bound to CD16, the FcR receptor for IgG, on the NK cell surface, triggering granule exocytosis and toxin secretion. NK cells are the principal mediator of this form of cytolysis, known as antibody-dependent cell-mediated cytotoxicity (ADCC) (1). The interaction with the FcR of NK cells may also evoke pleiotropic effects under permissive conditions, and is not restricted solely to the function of ADCC (17). NK cells can also lyse susceptible targets spontaneously by a second mechanism, which, although poorly understood, has generally been thought to occur without specificity and without MHC. Anti-IgG or anti-CD16 FcR antibodies which inhibit ADCC do not prevent NK killing (6), indicating that in this type of killing, target recognition is not mediated via the Fc receptor that functions in ADCC killing (18).

With the exception of the CD16 FcR used in ADCC, there is no definitive information yet on any type of receptor used by NK cells for target cell recognition and killing (6). Despite the fact that NK cells have a limited target cell range, no specific NK-associated surface receptor or target ligand has yet been defined (19). Although a molecular structure recognized by NK cells has not been defined, there is evidence that NK cells are capable of killing with specificity in a non-ADCC, non-MHC restricted manner.

The existence of NK specificity has been most simply demonstrated by the phenomenon of "cold target inhibition". In this

experimental situation one NK target cell type (unlabeled) can inhibit lysis of a different NK target type (labeled) by competing for effector cells (1). Cells that are not NK targets do not compete. Ciccone et al. first reported specific lysis of allogeneic cells by $CD3^-$, $CD2^+$, $CD56^+$ lymphocytes after their activation in a mixed lymphocyte culture in the presence of exogenous IL-2 (20). After a ten day coculture, these cells displayed cytolytic activity against PHA-induced blast cells bearing the stimulating alloantigens but not against autologous or unrelated allogeneic blast cells (20). Coculture with an autologous stimulator, or without IL-2, did not result in cytolytic activity directed at autologous or allogeneic cells, although cytolytic activity against K562 erythroleukemia cells (a classical NK target) was preserved in these controls. From these MLC-stimulated $CD3^-$ cells, clones were derived that lysed allogeneic lymphoid cells with apparent specificity, although the expression of CD16 and CD56 on these clones was not reported (19).

$CD3^-$, TCR^-, $CD16^+$ and/or $CD56^+$ NK lines and clones with apparent antigen specificity have been described (19). In addition to displaying potent cytolytic activity against K562 cells, these clones lysed their specific stimulator lymphoblastoid cell line (LCL) to a significantly to a greater extent than irrelevant LCL. This selective killing was inhibited by the addition of cold simulator LCL or K562 cells, or anti-LFA-1 mAbs, but not by irrelevant LCL or mAbs to CD3, class I or II MHC antigens (19).

The presence of a novel "antigen receptor" that may recognize a set of polymorphic endothelial cell antigens distinct from human leukocyte antigens was suggested by a study by Bender et al. They used cytotoxicity assays to demonstrate the ability of NK cells to lyse preferentially the line of microvascular endothelial cells to which they were previously bound for only 90-min (21). $CD3^-$, $CD16^+$ lymphocytes preferentially killed endothelial cells (ECs) with which they were cocultured over endothelial cells of unrelated donors (21). Antibodies blocking CD3, CD4, CD8, class I and II did not block killing, whereas anti-LFA-1 antibody consistently inhibited cytotoxicity, likely because of its role in leukocyte binding to ECs (21). Their results suggest that cytotoxic NK lines bind to their stimulating ECs

via both nonspecific and specific ligand interactions, and further suggest that the basis for their specific cytotoxicity may lie, at least in part, with specific binding (21).

As the membrane target structures have not been identified, it is not known whether different NK cells react with the same or with different target antigens (7). In fact, many studies have indicated that NK cells are not a homogeneous population in regard to expression of surface markers and cytotoxicity (22) but rather that subsets of NK cells recognize one or a limited number of target cells. Allavena and Ortaldo studied the heterogeneity among many clones of human NK cells with regard to phenotype and their patterns of cytotoxic reactivity towards a variety of tumor target cells (22). To determine whether the tumor specificity and surface antigenic profiles of NK cells are clonally distributed or whether individual clones of NK cells display the heterogeneity associated with the entire NK cell population, they tested the cytotoxicity of 196 clones of cultured LGL from three donors against a panel of tumor target cell lines, and found that the receptors recognized on NK-susceptible targets are clonally distributed among the LGL population. Interestingly, they also found that although most clones demonstrated both NK and ADCC killing, some other clones, which had NK activity, did not have ADCC, or vice versa. However, the cell surface phenotype of many of the LGL-derived clones included T cell markers, and the heterogeneity may represent different cell populations and not subsets of a single NK cell population.

NK cells isolated from individuals exhibit different allospecificities expressed by normal allogeneic target cells (23). Allavena and Ortaldo used their data from clones derived from purified preparations of LGL to support the hypothesis that the heterogeneity of the entire NK population is attributable to a mixture of clones that vary substantially in their target specificities and phenotypes (22). Regardless, while antigen-specific receptors have not been defined, and the molecular basis for such activity unknown, these studies indicate that NK cells do kill allogeneic cells selectively. Furthermore, this ability appears to be clonally distributed. In addition, the experimental data hint at the possibility of an NK cell

repertoire for the recognition of allogeneic cells, albeit a limited one in comparison to the T or B cell repertoire.

Now that it has been established that at least a subset of NK cells recognize allogeneic cells, the question posed is how is this specificity is achieved? Some research indicates that specific NK killing is MHC restricted. An inverse relationship between the expression of class I MHC antigens on target cells and sensitivity to NK reactions has been reported by several groups (3). Dawson et al. described studies in which infection of NK sensitive cells by strains of adenovirus known to decrease MHC class I molecule expression greatly enhances cytotoxicity (3). In general, target cell MHC class I antigen expression correlates with resistance to NK cell-mediated lysis (24). Thus, the potential role of MHC gene products as signal structures altering recognition by NK cells remains controversial (3).

Two models originally proposed to explain recognition of MHC class I deficient cells in murine hybrid resistance are applicable to the human alloreactive NK cell clones discussed above (25). They are called 'effector inhibition' and 'target interference'. In the 'effector inhibition' model, NK cell-surface molecules engage MHC class I antigens which consequently deliver inhibitory signals, requiring NK cell receptors that distinguish between different MHC class I alleles. In target interference, the NK cell receptors for specific antigens would not bind to target structures as they are masked by autologous MHC class I alleles. 'Target interference' requires that NK cell receptors recognize different non-MHC target structures, and that MHC class I alleles can distinguish and interact with different target structures.

Supporting the 'effector inhibition' model is the hybrid resistance phenomenon, the MHC-linked, NK cell mediated rejection of homozygous parental bone marrow grafts by heterozygous F_1 irradiated mice. Studying scid/scid mice challenged with H-2 homozygous bone marrow cells that are either Hh-1^+ or Hh-1^-, Murphy et al. presented evidence that these noncodominantly inherited histocompatibility determinants may be specifically recognized by NK cells, leading to graft rejection (3).

These models are not mutually exclusive and the 'masked' nonself determinant in the target interference model might be a polymorphic, MHC-encoded Hh antigen (25). In both models, a somatic, MHC-controlled determination of the NK cell receptor repertoire would be necessary for self-tolerance. This is supported by studies on human alloreactive NK cell clones, and by studies of hybrid resistance, H-2 transgenic and beta2-m knockout mice (25). The studies on human alloreactive NK clones, as well as mouse hybrid resistance and transgenic studies, stress the non-responsiveness to self and the reactivity to qualitatively MHC-mismatched targets (25). Studies of transgenic mice provided evidence that MHC class I genes regulate the NK repertoire of transplant recipients as well as the NK susceptibility of grafted cells (25). Bone marrow cells from gene "knockout" mice indicate that loss of MHC class I expression leads to NK sensitivity (25).

Karlhofer et al. reported that NK cells with a surface molecule (LY-49) that specifically interacts with the peptide-binding domains of the MHC class I alloantigen do not lyse cells with the MHC domain, despite efficient spontaneous lysis by Ly-49$^-$ effector cells (24). In order to characterize NK cell-mediated specificity, Ciccone et al. studied the target cells to determine that susceptibility to lysis is inherited in an autosomal recessive fashion (26). They found that lysis of normal allogeneic cells by NK cells can be clearly distinguished from conventional non-MHC restricted lysis of tumor cells (26). While cloned NK cells specifically lysed appropriate normal allogeneic cells, the lysis of tumor target cells was clearly unrelated to this phenomenon (26).

Even so, most research supports that hypothesis that specific NK killing is non-MHC restricted. Regarding antigens seen by NK cells, it is agreed that the expression of either class I or class II MHC gene products on target cells is not required for the development of cytotoxicity. Suzuki et al. showed that antibodies to HLA class I, or HLA-DR had no effect on the lysis of target by either NK killing (of K562) or specific NK killing (of stimulator lymphoblastoid cell line) (19).

What then, could be the nature of the structures expressed on non-MHC restricted NK cells conferring upon those cells the capacity to recognize their targets? Little is known about the molecules involved in non-MHC-restricted killing. Several theories have been postulated. First, non-MHC-restricted killing may be mediated by multiple cell interaction/adhesion molecules present on the cell surface interacting with their natural ligands on the target cell. Selectivity of NK cells might be determined by the relative expression of several cell surface molecules and receptors, with no single molecule playing a unique and essential role (6). Such molecules include CD11/CD18, CD2, and CD45 present on NK cells. Sufficient engagement of one or more of these molecules could result in NK cell activation and target cell death (27).

The surface molecules of the CD11/CD18 family appear to play important functional roles in NK cell killing. Patients with selective deficiency of either are deficient in NK cell activity, which is probably a consequence of inability to bind target (6). CD56 may play an accessory role as a mediator of nonspecific adhesion between allospecific NK effectors and their targets. However, given the existence of multiple isoforms, the variable expression of these isoforms on allospecific NK lines, and the failure of any of anti-CD56 mAbs to affect the lysis by NK cells of "nonspecific" targets, such as K562, the likelihood that CD56 plays an accessory role as a mediator of nonspecific adhesion is poor (2).

Laminin, which increases on the NK cell surface upon stimulation with IL-2, may act in prolonging lysis (6). Schwarz and Hiserodt demontrated that IL-2 activated NK cells which generate broadly reactive non-MHC restricted cytotoxicity express laminin-like molecules on their surface while cells not expressing these activities do not appear to express such structures (28). Furthermore, anti-laminin antibodies block cytotoxicity at a postbinding stage, without inhibiting NK-target cell interaction (6).

Another theory is that non-MHC restricted specificity may be conferred by NK cell-specific "receptor(s)" that interact with specific moieties expressed on NK-sensitive target cells. Non-MHC-restricted killing of K562 leukemia cells by CTLs could be inhibited by

antibodies to CD3/TCR, suggesting that these cells may recognize target via the CD3/TCR receptor (5). Although an NK target recognition structure has not been identified, several molecules have been described that are NK cell-specific and appear to modulate NK cell-mediated killing, including two on human NK cells (27).

Moretta et al. identified functional surface molecules which are expressed variably within NK cell clones and presence of one combination of these markers correlated with NK cell ability to mediate specific lysis of normal allogeneic cells (29). This study implicated the involvement of one molecule, EB6, in specific NK cell recognition. However, other NK clones possessing the EB6 molecule did not lyse the specific target, further complicating the interpretation of the molecule's significance.

Moretta et al. also described GL183, a cell surface molecule present on a phenotypically stable subset of NK cells whose cytolytic behavior is modulated by anti-GL183 mAb (9). However, GL183 probably acts more as an regulator of function and is less likely to be involved in alloantigen-specific recognition as it is invariant in structure (2) and is not associated exclusively with NK activity (18). CD2 and CD16, which have been shown to mediate signal transduction and activation of cytoxic mechanisms, are unlikely candidates for the same reasons (2) and that mAbs directed against them fail to inhibit recognition and lysis of NK-susceptible target cells (30).

Monoclonal antibodies specific for CD45 have been reported to inhibit NK cell lysis of selected tumor cells, suggesting a possible role in conferring NK specificity, however CD45 is not NK cell-specific, as it is expressed on the majority of hematopoietic cells (30).

CD56, of which at least four distinct isoforms have been found, exist variably on allospecific NK lines (2). Two mAbs, which by immunoprecipitation analysis revealed they recognized distinct molecular isoforms of CD56, were generated that bound selectively to the majority of $CD3^-$, $CD16^+$, $CD56^+$ lymphocytes. They inhibited the lysis of specific allogeneic target cells by a panel of alloreactive NK lines, without affecting the ability of these cells to kill the classical NK target K562 (2). The role of CD56 in NK killing is incompletely

understood, however, the finding of multiple CD56 isoforms on NK cells is consistent with the hypothesis that these isoforms constitute an allorecognition apparatus with limited heterogeneity. In studies by Nitta et al., however, the contribution of CD56 to NK cell cytotoxicity and binding could be demonstrated only when the target cells also express CD56 (31). This would limit the target cell population of CD56 mediated toxicity to neural and muscle cells, which contain the N-CAM isoform, and other CD56 containing tissues.

In the current study, I attempted to further characterize membrane antigens on the surface of NK cells which are involved in alloantigen target recognition. Previously this lab created NK cell lines and clones against RPMI8866, an immortalized B cell line, which preferentially lysed the stimulating B cell line and were minimally cytotoxic for a third party of autologous B cell targets (Bender, unpublished data). A monoclonal antibody called C8, also created in this lab, was specific for a surface molecule on RPMI8866 and, when incubated in cytotoxicity assays, blocked killing of the RPMI8866 target cell but not of K562. In fact, there were $CD8^-$, $CD3^-$, $CD16^+$, $CD56^+$ RPMI8866-stimulated NK clones whose lysis of RPMI8866 cells was 100% inhibited by monoclonal C8. From these data, it was concluded that RPMI8866 cells are specifically recognizable NK targets, and that the molecule recognized by C8 is a target ligand on the B cell which binds to a putative NK receptor.

My project was directed at proving the hypothesis that the NK cells generated by stimulation with these B cells do have a receptor structure that enables them to recognize and specifically lyse these targets. Murine monoclonal antibodies specific for $CD3^-$, $CD5^-$, $CD8^-$ NK cells were generated and screened for specificity to the activated NK cell and effect on NK cell behavior in cytotoxicity assays. Antibody binding was measured on fresh NK cells versus NK cells activated by co-culture with RPMI8866. Cytotoxicity assays compared the effect of antibody on NK killing of the specific target, RPMI8866 B cells, with general cytotoxicity of K562. The results support the hypothesis that NK cells have surface molecules which do confer specificity and, when blocked by antibody, inhibit alloantigen target recognition.

METHODS

Isolation of $CD3^-$, $CD5^-$, $CD8^-$, $CD16^+$, $CD56^+$ Lymphocytes. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation and were monocyte and B cell depleted by plastic adherence followed by passage over nylon wool columns. To obtain $CD3^-$, $CD5^-$, $CD8^-$ cells, cells devoid of monocytes and B cells were incubated with anti-CD3 (7D6) mAb for 20 min. at 4°C, washed twice, and applied to plastic petri dishes (Becton-Dickenson #1005) (which were precoated for at least twenty min. with 10 µg/ml goat anti-mouse IgG in Tris-HCl, pH 9.5, and washed with PBS) for 70 min. at 4°C. (panning). Nonadherent cells were retrieved by gently washing the plates with cold PBS 1% FBS. They were then incubated with anti-CD5 (S1.6) mAb and panned on anti-mouse IgG to remove any residual $CD5^+$ cells. Nonadherent cells were retrieved and incubated with anti-CD8 (3B5) mAb and panned on anti-mouse IgG to remove any residual $CD8^+$ cells. The resultant lymphocyte population contained >90% $CD56^+$ and <1% $CD3^+$ cells by flow cytofluorometric analysis.

Production of 1F7 and 1F8 mAbs. A 6-wk-old female BALB/c mouse was immunized with $CD3^-$, $CD5^-$, $CD8^-$, $CD16^+$, $CD56^+$ NK cells. Immunizations were performed using Ribi adjuvant (Ribi Immunochem Research, Inc., Hamilton, MT) according to the manufacturer's protocol. The immunization schedule consisted of 3 intraperitoneal injections of 10^7 of the relevant cells in 0.5 ml. PBS. After the third immunization, the mouse was sacrificed humanely by cervical dislocation and splenectomized. The sensitized splenocytes were fused with SP2/0 murine myeloma cells (non-Ig producer), thus creating hybrid cells which produce antibodies directed against the immunizing NK cells. The hybridomas were placed in a limiting dilution, and 1 cell/well was placed in 96-well round-bottom plates (Becton-Dickinson, Lincoln Park, NJ).

Culture medium. Standard culture medium consisted of RPMI 1640 (Biofluids, Inc., Rockville, MD or Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids), 1% (2mM) L-glutamine, and 1% streptomycin (100 μg/ml)/penicillin (100 U/ml). Medium for NK cell cocultures included 0.1% 2-Methionine (2-ME). Hybridomas were in HAT medium (standard plus 1% hypoxanthine-thymidine, 0.1% aminopterin) for first 2 weeks before being switched to HT (without aminopterin) and then standard. Hybridomas were grown in standard culture medium and split every 2 to 3 days.

RPMI8866 Coculture. Peripheral blood lymphocytes were isolated on Ficoll-Hypaque gradients, and spun once at each 2000, 900, and 700 rpms for 5, 10, and 15 min., respectively. The fast spin removed residual Ficoll, and the two slow spins removed platelets. Cells were brought up to a volume of 10^6 cells/ml in standard medium plus 2-ME. 1 ml was placed in wells of 24-well flat-bottomed multi-well plates (Becton-Dickinson, Lincoln Park, NJ). RPMI8866 stimulator cells at 4 x 10^5 cells/ml were Cs-irradiated at 6000 rads., and 0.5 ml was placed into each well. Cocultures were refed on days 7 and 10, and harvested on day 14, with about 70% $CD3^-$, $CD16^+$, $CD56^+$ cells by flow cytofluorometric analysis. Thereafter, the cocultures could be prolonged either in their wells or in bulk by refeeding with irradiated RPMI8866 stimulator cells at a ratio of 2 x 10^5 feeders: 10^6 NK cells on a weekly basis.

Screening of Hybridoma Supernatants. 166 hybridoma supernatants were screened on the basis of their ability to bind to activated $CD3^-$, $CD5^-$, $CD8^-$, $CD16^+$, $CD56^+$ lymphocytes by flow cytofluormetric analysis. 123 hybridoma supernatants were also screened for staining on RPMI8866 B cells and 99 were tested for antibody binding to $CD3^+$ T lymphocytes. 19 predominantly NK cell specific antibody secreting hybridomas were then evaluated in cytotoxicity assays with RPMI8866 targets. Of these, 2 antibodies were also tested in cytotoxicity assays with K562 as targets. When screening the hybridomas for antibody recognizing NK cells, the negative

control antibodies used were HPCG14 (murine irrelevant IgG1) and OKT3 (anti-CD3), and the positive control antibodies were W6/32 (anti-HLA class I) and 3G8 (anti-CD16). When screening the hybridomas for antibody recognizing B cells, the negative control antibody used was HPCG14 and the positive control was C8 (anti-RPMI8866 cells). When screening the hybridomas for antibody recognizing T cells, the negative control antibody used was 3G8 and the positive control antibodies were OKT3 and 7D6 (anti-CD3).

One and Two-color Flow Cytofluorometric Analysis. For surface marker analysis, 2 x 10^5 cells were stained with the saturating amounts of appropriate mAb, and if unconjugated, washed and stained with fluoresceinated goat anti-mouse IgG+IgA+IgM(H+L) Double Staining Grade (Zymed). Each incubation was for 25 min. on ice to prevent cell-surface modulation of Ag. All samples were placed in 0.35 ml of staining buffer (PBS 1% BSA 0.1% sodium azide). Events were collected on a flow cytometer (FACSort, Becton-Dickinson, Mountain View, CA), gated to exclude nonviable cells, and analyzed with FACScan Research Software (LYSYS II, Becton-Dickinson). Results are expressed as arbitrary normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity.

For two color staining, cells were stained with a FITC-conjugated antibody for 10 min. and then a PE-conjugated antibody for 15 additional min. before the cells were washed and placed in staining buffer. Controls for phenotyping the cells included cells stained with an irrelevant antibody bound to FITC and to PE.

The mAbs used in these and other studies were anti-Leu-4-FITC (anti-CD3, AMAC, Inc.), anti-Leu 11-FITC (anti-CD16, AMAC, Inc.), anti-CD56-PE (AMAC, Inc.), Irrel.-FITC (Sigma), Irrel.-PE (Sigma), HPCG14 (murine irrelevant IgG1), OKT3 and 7D6 (anti-CD3), S1.6 (anti-CD5), 3B5 (anti-CD8), W6/32 (anti-class I), 3G8 (anti-CD16), TS1.22 (anti-CD11a, anti-alpha subunit of LFA-1), C8 (anti-RPMI8866), anti-CD56.

In order to determine whether mAbs 1F7 or 1F8 block binding of anti-CD56 antibody, $CD3^-$, $CD16^+$, $CD56^+$ lymphocytes were treated

with 1F7 or 1F8 or anti-CD56 or 3G8 mAbs at 4°C for 30 min., washed and then stained with irrelevant PE second step to assess background blocking which was zero. The two test mAbs and anti-CD56 were incubated on separate NK cells, washed and stained with anti-CD56-conjugated PE second step and analyzed as above. (FITC=fluorescein isothiocyanate conjugated, PE=phycoerythrin-conjugated)

Supernatant Concentration. Selected supernatants were concentrated to 25x with Centriprep-30 Concentrators (Amicon, Inc., Beverly, MA). After prerinsing concentrator with deionized water, supernatant was placed in concentrator and centrifuged at 2800 rpm for 15 min. Filtered supernatant was removed and the concentrator was re-spun twice at 2800 rpm for 5 min. each. Retentate was removed with syringe and syringe-filtered into aliquots for storage.

Isotyping and Enzyme-Linked Immunosorbent Assay. Plastic ELISA plates were precoated with 1 µg/ml each of goat anti-mouse IgG and IgM in a coating buffer and incubated overnight at 4°C. Plates were washed twice with PBS 1% BSA 0.1% sodium azide and incubated overnight with PBS 3% BSA 0.1% sodium azide filling the wells. Plates were washed three times with .05%Tween-20. Test supernatants were added and incubated at 4°C 2 hours-overnight. Plates were washed three times with .05%Tween-20. Goat anti-mouse conjugated alkaline phosphatase second step was added and the plate incubated at least 2 hours at room temp. Plates were washed three times with .05%Tween-20 and twice with PBS 1% BSA 0.1% sodium azide. Disodium p-nitrophenyl phosphate (Sigma) substrate solution was added. Plates were read at 15 min. intervals by an ELISA-plate reader (Dynatech).

Isotyping of selected antibodies was done using an ELISA-based isotyping kit (Southern Biotechnology Assoc., Birmingham, AL).

Cytotoxicity assay. The most commonly used test of NK cell activity in vitro (assay for NK cell cytotoxicity) is the ^{51}Cr (sodium chromate)-release cytotoxicity assay, in which NK cell-containing cell

preparations are mixed with a constant number of ^{51} Cr-labeled K562 or other target cells at one or more effector-to-target cell ratios, and cell lysis is evaluated, after 3-4 hours of incubation at 37°C, by measuring the amount of ^{51}Cr released in the supernatant fluid (32).

Cytotoxicity of NK cells against RPMI8866 cells and K562 cells was measured in a 4 h ^{51}Cr-release microcytoxicity assay using 96-well U-bottomed microplates (Becton Dickinson, Lincoln Park, NJ). The target cells were labeled with 75 µCi of $Na_2{}^{51}CrO_4$ per 1.5 x 10^6 cells, washed, and seeded into 96-well culture dishes at 5 x 10^3 cells/well in suspension. Test and irrelevant isotype-matched control antibodies at the same approximate concentrations were placed in appropriate wells in order to explore the role of cell surface molecules in cytotoxicity. Suspensions of effector cells were added to quadruplicate wells to give various E/T ratios, ranging from 100:1 to 0.5:1, in a final volume of 200 µl. After an incubation at 37°C for 4 h, 150 µl of supernatant was removed from each well and counted in a gamma counter to determine experimental release (ER). Spontaneous release (SR) was obtained from wells receiving target cells and medium only, and maximum release (MR) was obtained from wells receiving 1% Triton X-100. The percent cytotoxicity was calculated by the following formula:

$$\text{cytotoxicity} = \frac{(ER)-(SR)}{(MR)-(SR)} \times 100$$

SR was 20% of max when RPMI8866 cells were used as a target. SR was 7.5% of max when K562 cells were used as a target. Experimental replicates consistently varied by <10%.

All work was performed by the author. Dr. Bender taught all experimental protocols and use of equipment, as well as assisting in early cytotoxicity assays and the running of FACSort samples in large experiments. Jean Wilson and Leslie Tacket instructed me on tissue culture technique and preparation of reagents.

RESULTS

Activation and proliferation of NK cells. Cell populations enriched in activated $CD16^+$, $CD56^+$ lymphocytes were obtained by coculture of PBLs with RPMI8866 cells for 2 wk. As shown in Figure 1A for a representative coculture, the resulting lymphocyte population was 70% NK cells and 30% T cells by flow cytometry. Purification of $CD3^-$, $CD8^-$, $CD16^+$, $CD56^+$ NK cells was achieved by panning for removal of $CD3^+$ T cells as described in Methods. Figure 1B shows a phenotype representative of the nonadherent cells from a panning after incubation with mAb 7D6 (anti-CD3), >99% $CD16^+$, $CD56^+$ NK and <0.5% $CD3^+$ T cells. Cells purified in this way were used in experiments requiring pure NK cell populations.

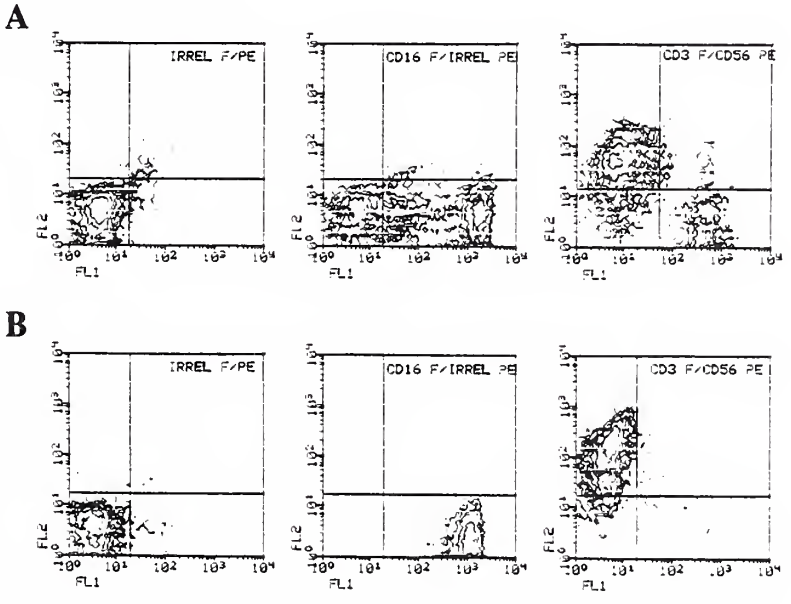

Figure 1. Two-color flow cytometric analysis of stimulated lymphocyte phenotype (**A**) after coculture of PBLs with RPMI8866 cells; (**B**) of nonadherent coculture cells following panning with anti-CD3 mAb. FL1, recorded in log scale on the x-axis, indicates the intensity of FITC staining. FL2, recorded in log scale on the y-axis, indicates the intensity of PE staining.

Evaluation of NK Cytotoxicity. To determine whether these $CD3^-$, $CD16^+$, $CD56^+$ NK cells had cytolytic activity, they were tested as effectors in 4 h ^{51}Cr-release assays against the original stimulator lymphoblastoid cell line, RPMI8866 as well as NK sensitive K562 cells. As shown in Figure 2, these cells exhibited potent lysis of both RPMI8866 and K562 cells which titers with the E : T ratio.

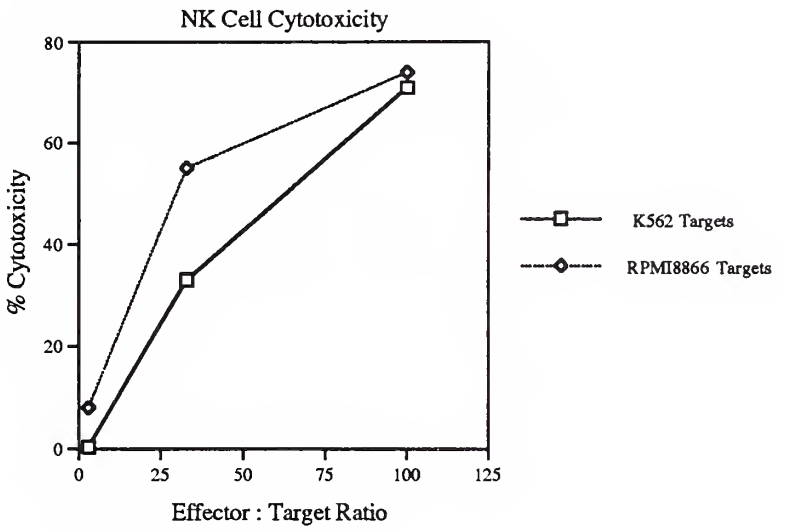

Figure 2. Cytotoxic activity of RPMI8866-stimulated $CD3^-$, $CD16^+$, $CD56^+$ NK cells was tested against the original stimulator RPMI8866 cells and K562 cells.

Expression of molecules on activated NK cells generated from peripheral blood. Among the 166 hybridoma supernatants tested against activated $CD3^-$, $CD8^-$, $CD16^+$, $CD56^+$ lymphocytes and analyzed by flow cytometry, 54 had antibody that bound to the NK cells. Of those antibodies, 37 also bound to molecules on the surface of B cells, and 19 also bound to molecules on the surface of activated $CD3^+$ T cells. Of the 54 NK-positive antibody secreting hybridomas, 1 produced antibody specific for NK cells with no staining of B or T cells (Figure 3), 3 which were negative on B cells but were slightly positive on T cells (Figure 4), 2 which were negative on B cells but very positive on T cells (Figure 5), 1 which was slightly positive on B

cells but negative on T cells (Figure 6), 3 which were slightly positive on both B and T cells (Figure 7), and 2 which were slightly positive on B and very positive on T cells (Figure 8). The rest were moderate to very positive on B cells, T cells, or both (data not shown).

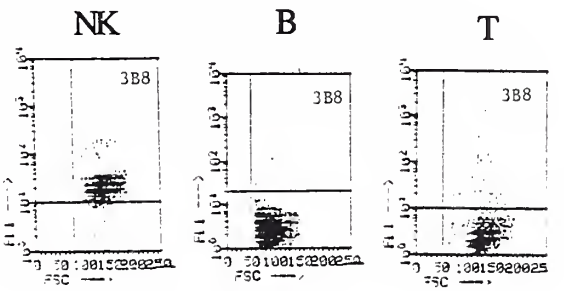

Figure 3. Staining of mAb 3B8 on NK, B, and T cells. mAb 3B8 recognizes a surface molecule unique to NK cells.

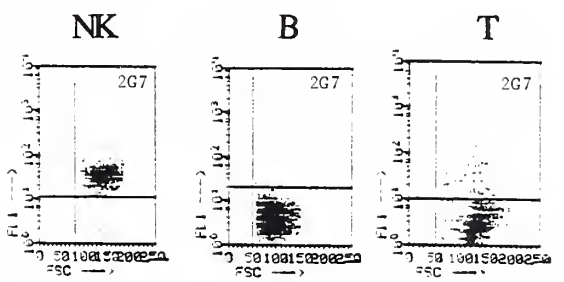

Figure 4. Staining of mAb 2G7 on NK, B, and T cells. This staining is representative of the three mAbs which recognize molecules which are present on NK cells and slightly on T cells, but absent on B cells.

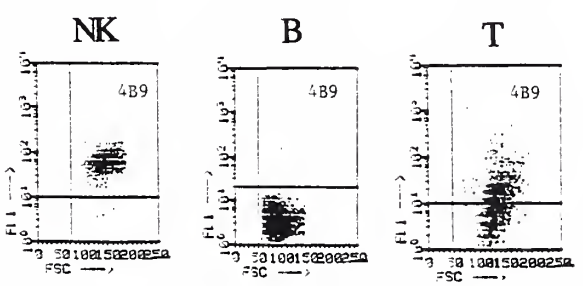

Figure 5. Staining of mAb 4B9 on NK, B, and T cells, which is representative of the two mAbs which recognize molecules on NK and T cells, and not on B cells.

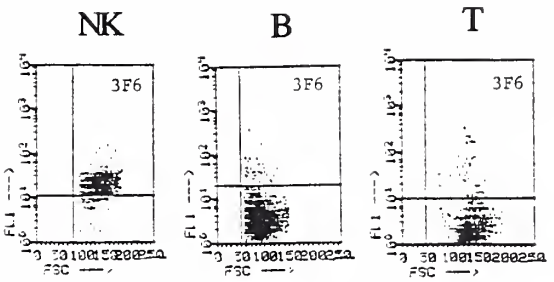

Figure 6. Staining of mAb 3F6 on NK, B, and T cells. The molecule recognized by mAb 3F6 is present on NK cells and a subset of B cells.

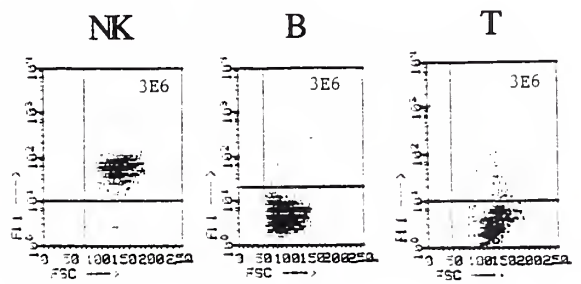

Figure 7. Staining of mAb 3E6 on NK, B, and T cells, which is representative of the mAbs which recognize a surface molecule on all three cell types.

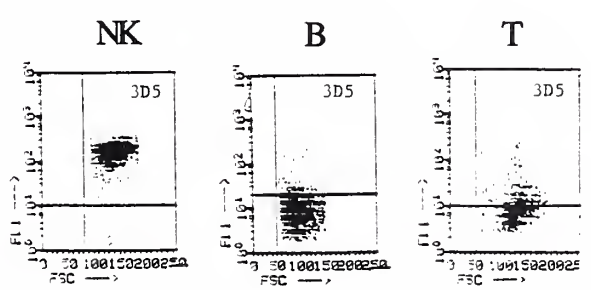

Figure 8. Staining of mAb 3D5 on NK, B, and T cells, which is representative of the two mAbs which recognize a surface molecule expressed highly on NK cells, moderately on T cells, and in low density on B cells.

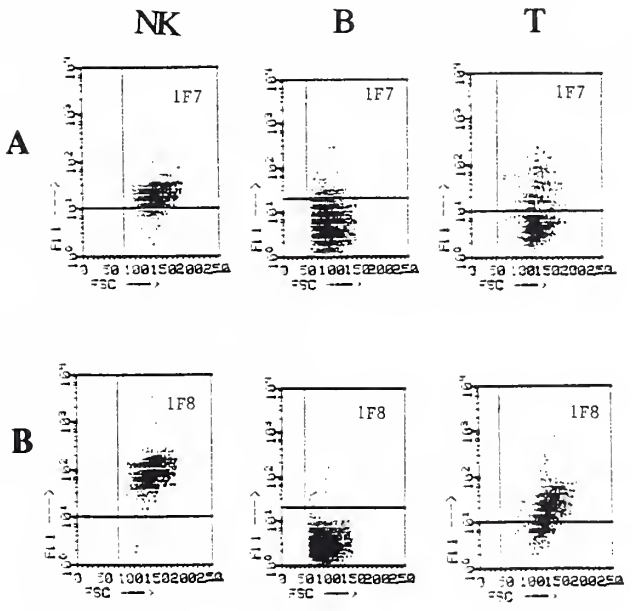

Figure 9. Staining of mAbs **A**) 1F7 and **B**) 1F8, which, from data obtained by following experiments, were found to be of interest. Initially, it was concluded that mAb 1F7 recognized a surface molecule on NK, B, and T cells. However, it was later determined that mAb 1F7 was of IgM isotype (data to follow). This makes its staining pattern somewhat difficult to interpret, because an irrelevent antibody of IgM isotype was not used in the early screening experiments. Thus, the small amount of reactivity with B and T cells may be a consequence of nonspecific binding. This requires further evaluation. Monoclonal 1F8 recognizes a surface molecule which is on NK cells and less on T cells, but not on B cells.

Figure 10A, B, and C shows the controls for the staining of $CD3^-$, $CD8^-$, $CD16^+$, $CD56^+$ NK cells, RPMI8866 B cells, and $CD3^+$, $CD16^-$ T cells, respectively as described in Methods.

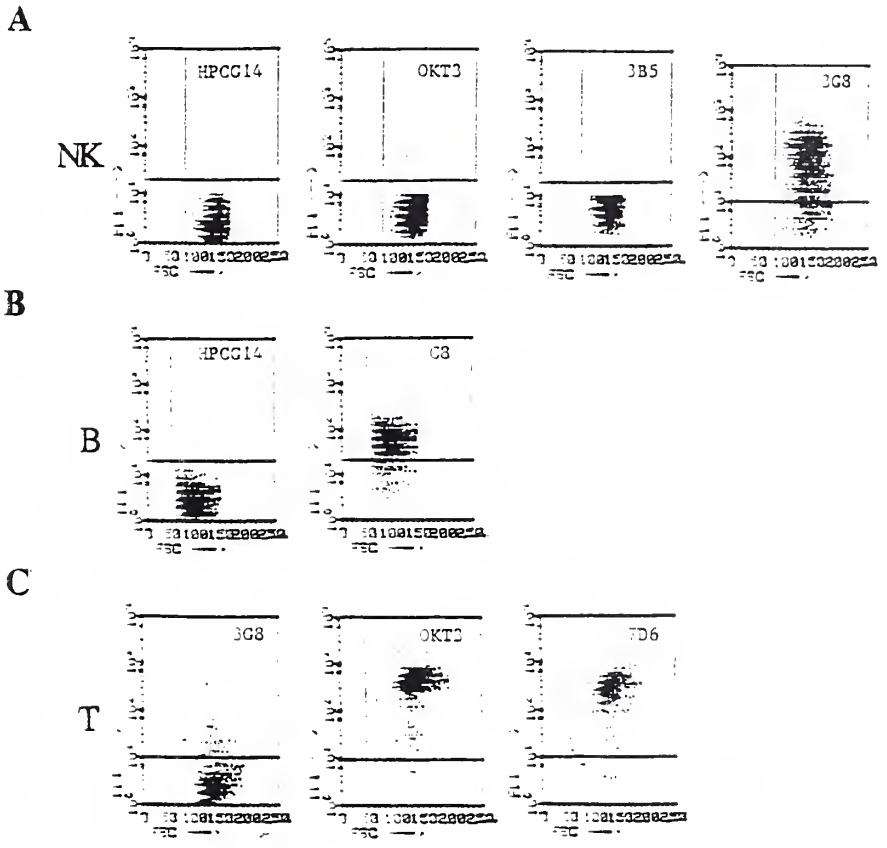

Figure 10. One-color flow cytometric analysis of cell surface antigens on three cell types. Positive and negative controls used for staining (**A**) NK cells; (**B**) B cells; and (**C**) T cells. In experiments using $CD3^-$, $CD8^-$, $CD16^+$, $CD56^+$ NK cells obtained as described in Methods, HPCG14, OKT3, and 3B5 were negative controls, while 3G8 was the positive control. For RPMI8866 B cells, HPCG14 was negative, while C8 was positive. For $CD3^+$, $CD16^-$ T cells, 3G8 was negative, while OKT3 and 7D6 were positive.

Isotyping of mAbs. Using an ELISA-based isotyping kit, mAb 1F7 and 1F9 were determined to be IgM, while mAbs 1E8, 1F8, 2G7, 3B8, and 3E6 were found to be of IgG1 isotype. The negative controls were standard medium and PBS 1% BSA 0.1% sodium azide. Positive control for IgG1 was mAb 3G8 (anti-CD16). Positive control for IgG2a was OKT3 (anti-CD3). Positive control for IgM isotype was mAb 1H12, an endothelial cell specific antibody. The results are shown in Table 1.

Table 1. Isotype of mAbs

	IgG1	IgG2	IgM
media	-0.006	-0.005	-0.006
1% BSA	-0.006	-0.005	-0.006
3G8	+0.083	-0.004	-0.003
OKT3	+0.004	+0.118	-0.005
1H12	-0.002	+0.032	+0.111
1E8	+0.090	-0.001	-0.001
1F7	+0.005	-0.001	+0.121
1F8	+0.069	-0.002	+0.001
1F9	+0.006	-0.002	+0.165
2G7	+0.160	+0.011	-0.004
3E6	+0.069	-0.002	+0.004

In experiments requiring isotype-specific controls for mAbs 1F7 and 1F8, mAbs 1H12 and 2G7 were used, respectively.

Functional role of molecules bound by mAb in cytotoxicity by activated NK cells. The functional involvement of molecules bound by these mAbs was tested. $CD3^-$, $CD16^+$, $CD56^+$ NK cells were tested for cytotoxic activity against RPMI8866 cells in the presence and absence of mAbs. The results of these experiments are shown in Figure 11, A and B. Although cytotoxic activity was high against the target tested, killing of the RPMI8866 target was inhibited in the presence of mAbs 1F7 and 1F8 at the 100:1 and 33:1 E:T ratios. mAb 1F7 blocked 14% of killing at the 100:1 ratio and 54% of killing

at the 33:1 ratio. mAb 1F8 blocked 13% of killing at the 100:1 ratio and 36% of killing at the 33:1 ratio.

No significant effects on RPMI8866 target cell killing were noted when using the other antibodies also reactive with $CD3^-$, $CD16^+$, $CD56^+$ NK effector cells (mAbs 3D7, 1F9, 4B9, 3F6, 3E6, 4D10, 3D5, 3E2) or in the presence of the isotype matched controls (shown in figures). Anti-LFA-1 antibody was used as a positve control which blocks killing by interfering with target cell adhesion. This screening procedure led to the identification of two hybridomas which secrete antibodies (mAbs 1F7 and 1F8) which inhibit the cytolytic activity of the NK cells against their original stimulator RPMI8866.

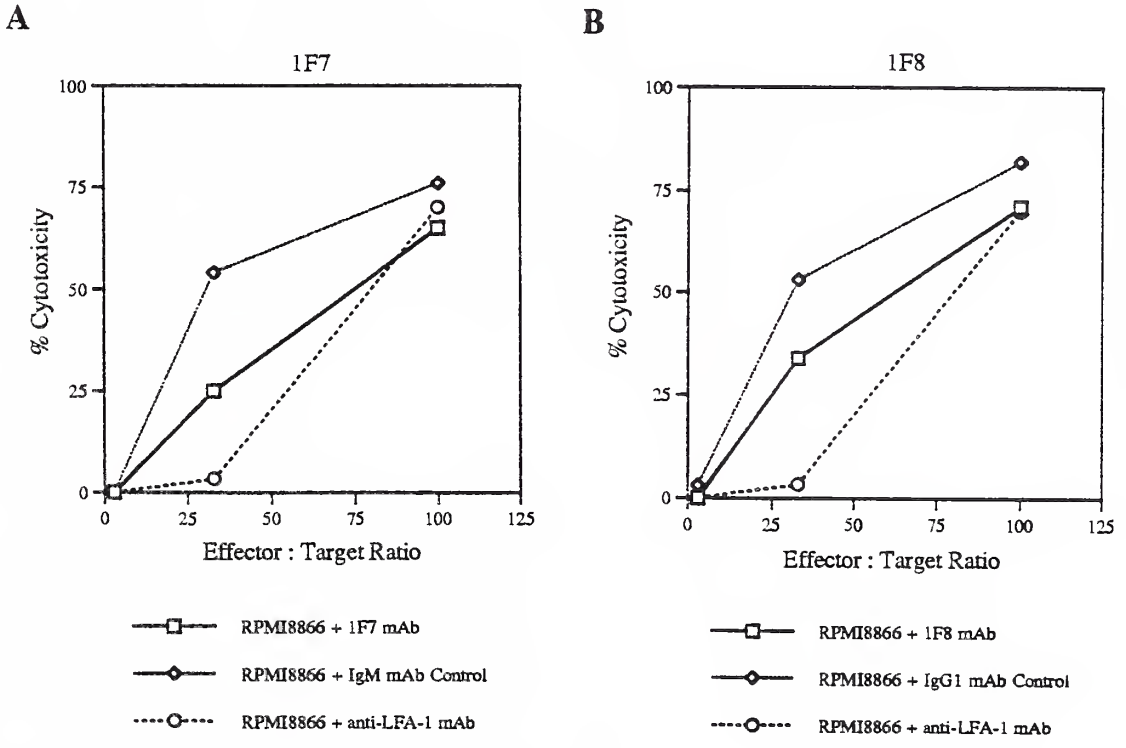

Figure 11. Partial inhibition of NK cell cytotoxicity of RPMI8866 stimulator cells by mAbs (**A**) 1F7 and (**B**) 1F8.

Demonstration of Allogeneic target recognition. In order to determine whether these mAbs were blocking NK killing in general, as opposed to allospecific killing, cytotoxicity assays were carried out with both RPMI8866 cells and K562 cells as targets in the presence of the mAbs and appropriate control mAbs. Although significant inhibition was achieved against killing of the RPMI8866 target as shown in Figure 11, the same antibodies at the same concentration did not significantly block killing of K562 at any of the effector : target ratios (Figure 12, A and B). This led to the identification of two hybridomas which secrete antibodies (mAbs 1F7 and 1F8) which inhibit the cytolytic activity of the NK cells against their original stimulator RPMI8866 but not of K562. That is, there appears to be a specificity to the antibody inhibitory effects.

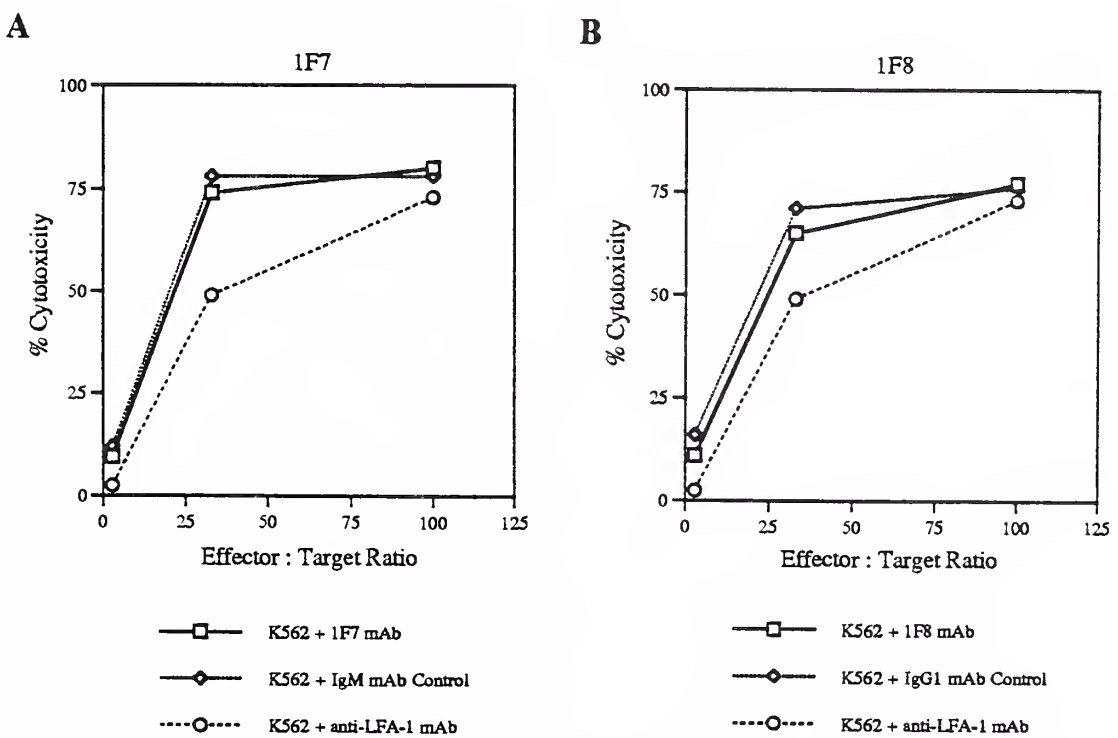

Figure 12. No effect on NK cytotoxicity of classical NK target K562 by mAbs (**A**) 1F7 and (**B**) 1F8.

Are mAbs 1F7 and 1F8 recognizing CD56? Once functional significance was established for the surface molecules recognized by mAbs 1F7 and 1F8, more information regarding what they may be was desired. A flow cytometric experiment was completed in an attempt to rule out the possibility that mAbs 1F7 and 1F8 are recognizing isoforms of CD56, as anti-CD56 mAbs have failed to affect the lysis by NK cells of "nonspecific" targets, such as K562, while inhibiting the lysis of specific allogeneic target cells by a panel of alloreactive NK lines (2).

In this experiment, mAbs 1F7 and 1F8, if not recognizing CD56, should have no effect on the staining of anti-CD56 PE or irrelevant PE. As a positive control, CD56 should block the staining of anti-CD56 PE without blocking irrelevant PE. As seen in Figure 13, anti-CD56 did not block the binding of anti-CD56 PE. Three possibilities explain this. First, the titer of the anti-CD56 was not known, and the amount used may have been too low to saturate cell surface molecules. Second, as previously discussed, there are multiple isoforms of CD56. These mAbs came from different hybridomas and may be recognizing different isoforms. Third, within isoforms, there are multiple non-crossblocking epitopes that these different mAbs could be recognizing.

Monoclonal antibody 1F8 did not affect the binding of anti-CD56 PE. Monoclonal antibody 1F7, however, appears to have decreased the binding of anti-CD56 PE, lowering its mean fluorescence from >100 to <50. This may be due to nonspecific blocking of the anti-CD56 PE, and may be a result of the bulky pentameric structure of the 1F7 IgM antibody. Since CD56 is present only on NK cells and a minor subset of cytotoxic T cells, it is unlikely that mAb 1F8 recognizes CD56 on these cells as the molecule it recognizes are present at low densities on T cells (Figure 5). Furthermore, neither the RPMI8866 or K562 target cells express CD56, a requisite if CD56 mediated cytotoxicity is assumed to be the cause, according to the previously mentioned studies by Nitta et al. (32). This requirement, however, is disputed by Suzuki et al. (2).

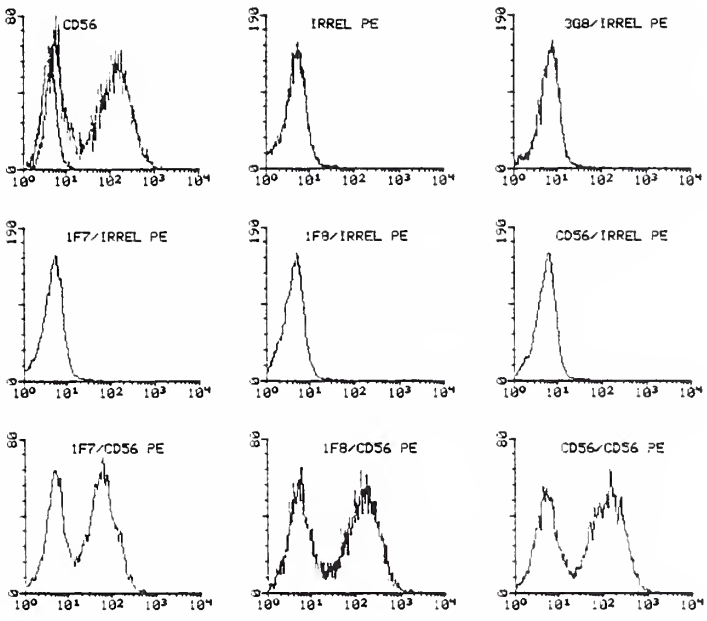

Figure 13. Monoclonal antibody blocking experiment. NK cells were treated with mAbs 1F7, 1F8, or CD56, before treatment with, in one sample, irrelevent PE and, in another sample, CD56 PE.

DISCUSSION

NK lymphocytes have the ability to lyse target cells without apparent prior sensitization or MHC gene restriction (1-4). Despite the fact that NK cells have a limited target cell range, no specific NK-associated surface receptor or target ligand has yet been defined. Traditionally, it has been concluded that these cells, in contrast to T or B cells, lack a highly refined antigen recognition system, and are only nonspecific effectors. However, scrutiny of NK cytotoxic activity reveals heterogeneity of both NK recognition and target structrures (33). In addition, murine NK cells have been shown to mediate the genetically restricted hybrid resistance to parental bone marrow allografts (3), a phenomenon that appears directed at the products of noncodominant hematopoietic histocompatibility genes. More recently, evidence has been provided that human NK cells are capable of alloantigen recognition (19,20). Therefore, on the basis of these observations it would appear that, contrary to previous opinion, at least a subset of NK cells have the ability to distinguish target cells.

The identification of surface structures involved in target cell recognition by NK cells has become an area of active investigation. Candidate markers must display a distribution restricted to functionally relevant effector cells, and antibodies directed against such putative receptors must alter the cytotoxic activity of such cells (3). In the present study, using NK cells stimulated with the NK-sensitive RPMI8866 B cell line as an immunogen, two mAbs (1F7 and 1F8) were generated which inhibit lysis of RPMI8866 cells by $CD3^-$, $CD16^+$, $CD56^+$ NK cells without affecting the ability of these cells to kill the classical NK target cell K562.

Double fluorescence and FACS analysis showed that $1F7^+$ and $1F8^+$ cells were consistently included in the $CD3^-$, $CD16^+$, $CD56^+$ cell populations. 1F7 mAb recognizes a surface molecule on these NK cells, and only questionably on B or T cells. The molecule recognized by mAb 1F8 is on NK and a percentage of T cells, while absent on B cells. It is not known if the $1F8^+$ T cells detected in activated cultures arise from expansion of a small number of positive cells

present in the peripheral blood, and/or if the molecule(s) is induced by activation of previously negative T cells.

What do these mAbs recognize, and are they truly novel? Given that mAbs 1F7 and 1F8 inhibit target cell lysis, it is unlikely that they recognize CD2 or CD16, since both anti-CD2 and anti-CD16 mAbs enhance NK-cell mediated killing (9,30). Studies using mAb against CD8, a surface structure shared by some NK cells and T cells, have failed to alter the cytotoxic activity of NK cells or the activity of these cells after activation by IL-2 (28). Unlike the molecules recognized by mAbs 1F7 and 1F8, CD2 is present on all T cells. Additionally, the patterns of antibody staining by anti-CD16 and anti-CD8 differ from that of both anti-1F7 and anti-1F8 (Figures 9 and 10). Monoclonal antibodies specific for CD45 have been reported to inhibit NK cell lysis of selected tumor cells, however, as it is expressed on virtually all hematopoietic cells except mature erythrocytes and their immediate precursors (30), it is unlikely to be recognized by mAbs 1F7 or 1F8. Monoclonal antibodies 1F7 and 1F8 clearly do not recognize CD11/CD18 (LFA-1), because they do not block killing of K562 and, again, are subset-restricted.

Immunofluorescence analysis of the NK cells could not refute the possibility that the antigenic epitopes recognized by these two mAbs are closely associated with, if not identical to CD56. Although mAb 1F8 does not block the staining pattern of anti-CD56 mAb, it is possible that it recognizes a non cross-blocking epitope. This may also be the case for mAb 1F7. It does affect the anti-CD56 mAb staining, but it is unclear that this effect is specific and not the result of non-specific blocking by a large IgM antibody. Using an irrelevent IgM antibody in this experiment would resolve this issue. The question of whether these mAbs recognize and block an epitope on NK CD56 is interesting because of the controversy regarding the function of CD56, and its significance with respect to the NK-target cell interaction.

In the study by Nitta et al., CD56 was reported to be involved in cytotoxic activity and only homotypic adhesion between NK cells and $CD56^{+}$ malignant neural cells, because targets without CD56 were not susceptible to lysis by NK cells (31). In contrast, results obtained

by Suzuki et al. suggests that CD56, of which they have detected at least four isoforms, is directly involved in NK cell lysis of alloantigen-specific targets (2). In their study, the lymphoblastoid cell line targets lacked any detectable expression of CD56, indicating that the CD56 isoforms on the NK cell effectors mediate a heterotypic rather than a homotypic interaction between NK cells and their targets. They used the finding of multiple CD56 isoforms on NK cells, the variable expression of these isoforms on allospecific NK lines, and the failure of any of their anti-CD56 mAbs to affect the lysis of NK cell of "nonspecific" targets such as K562 cells, to conclude that the CD56 isoforms constitute an allorecognition apparatus.

There is considerable information still to be obtained regarding these molecules. Plans for the future include: 1) Testing absolute specificity for the stimulator cells. Cold target inhibition studies could be done to confirm the specificity of the NK cells for the RPMI8866 stimulators. To prove that they truly block allospecific killing would require that they be tested with NK cells against a panel of target cells. 2) Phenotyping of additional cells for surface antigens recognized by mAbs 1F7 and 1F8. For example, staining freshly isolated T cells would answer the question of whether the $1F8^+$ T cells detected in activated cultures arise from expansion of a small number of positive cells present in the peripheral blood, and/or if the molecule(s) is induced by activation of previously negative T cells. Generating NK clones and screening them for recognition by these mAbs would provide more information regarding heterogeneity of expression of the molecules they recognize. 3) Evaluating the possible role of molecules recognized by mAbs 1F7 and 1F8 in signal transduction. This can be accomplished by measuring Ca^{2+} fluxes resulting from mAb crosslinking. Assessing the ability of NK cells to specifically lyse the hybridoma cells that produce antibodies (mAbs 1F7 and 1F8) against a candidate triggering molecule on the effector cells, known as reverse ADCC, can also be used as a sensitive test for the ability of the molecule to transduce signals into the cell. For example, cytotoxic T cells specifically lyse anti-CD3 mAb-producing hybridomas, and human NK cells lyse anti-CD16 mAb-producing hybridomas (27) and anti-

CD56 mAb producing hybridomas (2). 4) Biochemistry. Immunoprecipitation and/or western blotting would be necessary to assess the molecular mass and extent of glycosylation of the surface antigens recognized by these mAbs. In order to conclude with certainty that mAbs 1F7 and 1F8 do or do not recognize CD56, their target ligand(s) would need to be immunoprecipitated and further characterized. And ultimately, 5) expression cloning from an RPMI8866-stimulated NK cell cDNA library, 6) sequencing, 7) transfection of cDNA into cells lacking NK activity, and 8) abrogation of expression of proposed cDNA in NK cells with antisense cDNA vectors.

With this information in hand, it would be clarified whether the molecules recognized by mAbs 1F7 and 1F8 play a primary (receptor) or a secondary (accessory) role in antigen-specific killing. One would predict, if a molecule is a receptor involved in recognition and mediation of cell death by NK cells, that: 1) it would be present only on those cells that mediate this function; 2) absence of the molecule would be associated with lack of function; 3) the engagement of the molecule by its antibody would modulate its function; 4) affinity purified antibodies which block target killing could do so without disrupting primary target cell adhesion; 5) transfection of specific cytotoxic cells with the gene or cDNA of the molecule would enable them to mediate non-MHC-restricted killing; and 6) the ligand for the receptor would be expressed on appropriate target cells (27,28).

At present it is only known that the molecules recognized by mAbs 1F7 and 1F8 fulfills two of these criteria. They are present only on those cells that lyse NK-sensitive targets and this killing is partially inhibited, when engaged by their respective antibodies. Taken together, these data suggest that 1F7 and 1F8 surface molecules may exert a role in the activation or regulation of the cytolytic function of human NK cells. The antigenic determinant recognized by 1F7 or 1F8 mAb could represent a "receptor" molecule uniquely expressed on NK cells. Determining whether either molecule is a true NK recognition receptor will require additional confirmation either by expression cloning, sequencing, and

transfection of a full-length cDNA into cells lacking NK activity or by abrogation of expression of the proposed receptor in NK cell lines or clones with antisense cDNA vectors.

ENDNOTES

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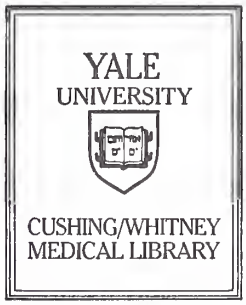
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